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Operant ethanol self-administration increases extracellular-signal regulated protein kinase (ERK) phosphorylation in reward-related brain regions: selective regulation of positive reinforcement in the prefrontal cortex of C57BL/6J mice

Sara Faccidomo[1], **Michael C Salling**[1], **Christina Galunas**[1], and **Clyde W Hodge**[1,2]

[1]Bowles Center for Alcohol Studies, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

[2]Department of Psychiatry, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

Abstract

Rationale—Extracellular-signal regulated protein kinase (ERK1/2) is activated by ethanol in reward-related brain regions. Accordingly, systemic inhibition of ERK1/2 potentiates ethanol reinforcement. However, the brain region(s) that mediate this effect are unknown.

Objective—To pharmacologically inhibit ERK1/2 in the medial prefrontal cortex (PFC), nucleus accumbens (NAC) and amygdala (AMY) prior to ethanol or sucrose self-administration, and evaluate effects of operant ethanol self-administration on ERK1/2 phosphorylation (pERK1/2).

Methods—Male C57BL/6J mice were trained to lever press on a fixed-ratio-4 schedule of 9% ethanol+2% sucrose (ethanol) or 2% sucrose (sucrose) reinforcement. Mice were sacrificed immediately after the 30th self-administration session and pERK1/2 immunoreactivity was quantified in targeted brain regions. Additional groups of mice were injected with SL 327 (0–1.7 μg/side) in PFC, NAC or AMY prior to self-administration.

Results—pERK1/2 immunoreactivity was significantly increased by operant ethanol (g/kg=1.21 g/kg; BAC=54.9 mg/dl) in the PFC, NAC (core and shell), and AMY (central nucleus) as compared to sucrose. Microinjection of SL 327 (1.7 μg) into the PFC selectively increased ethanol self-administration. Intra-NAC injection of SL 327 had no effect on ethanol- but suppressed sucrose-reinforced responding. Intra-AMY microinjection of SL 327 had no effect on either ethanol- or sucrose-reinforced responding. Locomotor activity was unaffected under all conditions.

Conclusions—Operant ethanol self-administration increases pERK1/2 activation in the PFC, NAC and AMY. However, ERK1/2 activity only in the PFC mechanistically regulates ethanol self-administration. These data suggest that ethanol-induced activation of ERK1/2 in the PFC is a critical pharmacological effect that mediates the reinforcing properties of the drug.

Correspondence: Dr. Clyde W Hodge, Bowles Center for Alcohol Studies, University of North Carolina at Chapel Hill, CB #7178, Thurston Bowles Building, Chapel Hill, NC 27599, Voice: 919-843-4389, Fax: 919-966-5679, clyde_hodge@med.unc.edu.

Keywords

ALCOHOL; REINFORCEMENT; ERK; MAPK; OPERANT CONDITIONING; ADDICTION; MOTOR ACTIVITY; PREFRONTAL CORTEX; ACCUMBENS; AMYGDALA

Introduction

Alcoholism is a progressive, debilitating and widespread threat to public health; about 30% of the US population battles chronic and disabling relationships with alcohol throughout their lifetime (Grant et al. 2004; Hasin et al. 2007). Given the heterogeneous nature of the disorder, it is not surprising that a “one drug fits all” treatment approach has limited effectiveness (Clapp 2012; Mann 2004). One strategy for developing effective pharmacotherapies is to understand the mechanisms that underlie the positive reinforcing effects of ethanol (Grant and Samson 1985; Meisch and Thompson 1973; Samson et al. 1988). Ethanol self-administration is known to be regulated by a number of neurotransmitter (Cannady et al. 2013; Hodge et al. 1993) and neuropeptide (Arolfo et al. 2009; Schroeder et al. 2003) receptor systems, and attention has focused on elucidating the downstream cell signaling pathways that modulate the reinforcing properties of ethanol (Besheer et al. 2012; Cui et al. 2013; Faccidomo et al. 2009; Jeanblanc et al. 2013).

ERK1/2 is a serine-threonine kinase and member of the larger mitogen-activated protein kinase (MAPK) family. When phosphorylated, the active form of ERK (pERK1/2) can influence many diverse neural processes including induction of transcription factors and immediate early genes (Davis et al. 2000; Vanhoutte et al. 1999), modulation of receptor insertion, trafficking and signaling (Besnard et al. 2014; Qin et al. 2005) and modifications of scaffolding proteins such as PSD-93 to alter dendritic spine density and neural transmission (Guo et al. 2012). These widespread consequences of ERK1/2 phosphorylation reflect ongoing neuronal plasticity that has been shown to be involved in drug-induced plasticity (Adams and Sweatt 2002; Davis et al. 2000; Girault et al. 2007) and might contribute to the development of escalated ethanol intake and addiction (Chandler and Sutton 2005; Zamora-Martinez and Edwards 2014).

Given that ethanol impairs normal brain function and plasticity, it is not surprising that ERK1/2 phosphorylation is altered by both acute and chronic ethanol exposure (Agoglia et al. 2015; Goulding et al. 2011; Spanos et al. 2012; Zhu et al. 2013) and during alcohol withdrawal (Cui et al. 2011; Pandey et al. 2008; Sanna et al. 2002; Valjent et al. 2001). Importantly, pERK1/2 is abundantly expressed in brain regions that modulate the rewarding effects of ethanol, including the nucleus accumbens (NAC), amygdala (AMY) and prefrontal cortex (PFC), and is up or down regulated after acute ethanol exposure in a sub-nuclei dependent manner (Ibba et al. 2009; Pandey et al. 2008; Rimondini et al. 2002; Rosas et al. 2014; Valjent et al. 2004). For example, an acute injection of 3 g/kg ethanol significantly increases ERK1/2 phosphorylation in the shell of the NAC, but decreases ERK1/2 phosphorylation in the core of the NAC (Agoglia et al. 2015). Likewise, multi-directional expression patterns of pERK1/2 are also observed in sub-regions of the amygdala after acute ethanol injection (Spanos et al. 2012).

Pharmacological inhibition of ERK1/2 phosphorylation has been shown to disrupt multiple measures of drug (Valjent et al. 2000; Zhai et al. 2008) and ethanol (Besheer et al. 2012; Faccidomo et al. 2009; Schroeder et al. 2008) reward including self-administration, reinstatement, conditioned place preference and locomotor sensitization. Interestingly, both the acquisition and expression of conditioned place preference to ethanol is not disrupted by ERK1/2 inhibition suggesting that the specific neural mechanisms that regulate the contextual learning that is required for ethanol conditioned place preference may be different from those involved in ethanol self-administration and reinforcement (Groblewski et al. 2011). Nonetheless, these studies provide converging lines of evidence to support the hypothesis that the MAPK/ERK signal transduction pathway is functionally involved in ethanol-induced neuronal plasticity and that disruption in this pathway, especially in reward- and memory-related brain regions, contribute to the development and persistence of addiction, dependence and relapse to alcohol (Radwanska et al. 2007; Self 2004).

Our laboratory has shown that systemic inhibition of ERK1/2, using the MEK inhibitor SL 327, produces a robust and systematic increase in operant responding for a sweetened ethanol solution in C57BL/6J mice (Faccidomo et al. 2009). It is notable that this increase in self-administration is specific to ethanol as a reinforcer, and does not generalize to other non-drug reinforcers, such as sucrose, or to other MAPK pathways. However, these studies do not address the relative contribution of specific brain regions on the alterations in ethanol self-administration seen after systemic SL 327 treatment. Given that ethanol can both increase and decrease pERK1/2 expression in a regionally-dependent manner, the primary objective of this study was to identify key brain regions that are functionally involved in MAPK regulation of ethanol reinforcement. To address this question, the present study first evaluated the effects of operant ethanol self-administration on pERK1/2 levels in the PFC, NAC, and AMY. Second, the MEK inhibitor SL 327 was infused site-specifically in the PFC, NAC and AMY immediately prior to self-administration sessions of an ethanol or a sucrose only solution to determine if ERK1/2 activity in these brain regions mechanistically regulates the positive reinforcing effects of ethanol.

Materials and Methods

Subjects

Subjects were male C57BL/6J mice (n=148) arriving at 10 weeks of age from Jackson Laboratory (Bar Harbor, ME). Mice were group-housed in clear, polycarbonate cages lined with corn-cob bedding. Each cage was covered by a stainless steel wire lid through which Purina rodent chow and water was available *ad libitum*. The vivarium was maintained on a 12h:12h reverse light/dark cycle (lights off at 0800) with temperature at 21 ± 1 °C. All procedures were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill and animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (2011).

Ethanol Self-administration Apparatus and Procedure

Self-administration sessions were conducted in 16 operant conditioning chambers (Med Associates, St. Albans, VT) that were each housed within a sound-attenuating cubicle

equipped with a 28V fan to provide ventilation and mask external noise. Opposite walls of the chamber each contained a stainless steel ultra-sensitive retractable lever located directly below a cue light. Responding on only one of the levers (the “active” lever) was reinforced. Responses on the “inactive” lever were recorded but held no contingencies. A drinking trough was located adjacent to the active lever and was connected to a syringe pump. Reinforcement delivery was accompanied by a brief illumination of the cue light and pump sound, both of which served as secondary cues. Responses during this time (800ms) were measured but did not contribute towards the response requirement (time-out period). The chamber and pump were connected to an interface and computer that recorded the input and behavioral output of each mouse (MED-PC for Windows v.4.1).

One week after arrival, mice were presented with either a 9% Ethanol (v/v) + 2% Sucrose (w/v) (ethanol) or 2% Sucrose (w/v) only (sucrose) solution, along with water, in their home cage for 2 weeks. This procedure was conducted to familiarize the mice to the reinforcing solution. We have found that this procedure generates high levels of ethanol self-administration that exceed the minimum criteria set for binge drinking by the NIAAA (see Faccidomo et al. 2009 for a representative blood alcohol content correlation). A slightly sweetened ethanol reinforcing solution was used because this reinforcing solution leads to more reliable and consistent intake as compared to unsweetened ethanol. Under these experimental conditions, mice consume the entire volume that is delivered and dissociation between the lever press and consummatory behavior is not observed.

After 2 weeks of home-cage self-administration, mice were placed into an operant conditioning chamber for a 16 hr overnight session during which they performed an operant response (lever press) that was reinforced with a delivery of either 9% ethanol + 2% sucrose (ethanol) or 2% sucrose only (sucrose) into the drinking trough. The reinforcement volume was set at .014ml. To facilitate acquisition of lever pressing, mice were water restricted for 23 hrs prior to the first 2 overnight sessions. During the first overnight session, mice responded on a Fixed-ratio 1 (FR 1) schedule of reinforcement. During the 2nd session, the response requirement was increased from FR 1 to FR 2 to FR 4 in increments of 25 (ie: after receiving the 25th reinforcement, the response requirement increased to FR 2, etc.). The third and subsequent sessions were shortened to 1 hr in duration and the response requirement was maintained at FR4. All 1 hr sessions were conducted in the dark, between 0900–1500h, 6 days/week. At the end of each session, the drinking trough was checked to verify consumption of all delivered fluid.

Immunohistochemistry

After the end of the 30th self-administration session, ethanol (n=8) and sucrose (n=8) self-administering mice in **Experiment 1** were deeply anesthetized with pentobarbital (150 mg/kg) and were intracardially perfused with ice cold phosphate buffered saline (PBS; 0.1M) followed by 4% paraformaldehyde (PFA). Blood was immediately collected before infusion of PBS for analysis of plasma blood alcohol content using an AM1 Alcohol Analyzer (Analox, Instruments, Lunenburg, MA). Brains were post-fixed in paraformaldehyde for 48 hrs and stored in PBS at 4°C until slicing. Coronal brain sections

(40μM) were sliced with a vibratome (Leica VT1000S, Leica Microsystems, Buffalo Grove, IL) and were stored at −20°C in cryoprotectant until use.

For measurement of pERK1/2 immunoreactivity a two day protocol was used. Free-floating tissue sections were washed with 0.1M PBS before being gently bathed in 1% H_2O_2 to inhibit endogenous peroxidase activity. Antigen retrieval was performed by immersing tissue in Citra buffer (1X; Biogenics, Napa, CA) for 30 min at 70°C. Sections were blocked with 5% normal goat serum (Vector Labs, Burlingame, CA) in PBS with 0.1% Triton X (PBS_{Tx}) for 1 hr before incubation with a primary antibody for pERK1/2 (1:400; anti-rabbit phospho44/42 MAPK [ERK1/2] antibody, #9102, Cell Signaling Technology, Danvers, MA) overnight at 4°C. On day 2, sections were washed in PBS_{Tx} and then incubated with a goat anti-rabbit secondary antibody for 1 hr at 24°C. Antibody bound pERK1/2 was visualized using a DAKO-DAB chromogen (Agilent Technologies, Carpinteria, CA), sections were counterstained with Toluidine Blue and were mounted onto slides for analysis.

Quantification of immunopositive pixels and cells was conducted at 20X using a light microscope (Olympus CX41, Olympus, Tokyo) connected to a computer which ran Bioquant software (Bioquant Life Science Software v.8.40.20; Bioquant Corp, Nashville, TN). A digital camera (Regita OEM fast, QImaging, Burnaby, BC) attached to the microscope was used to acquire all images. The number of immunoreactive pixels and immunopositive cells were measured for the medial prefrontal cortex (AP range: +1.98 to +1.70), the core and shell of the nucleus accumbens (AP range: +1.54 to +1.18) and the central and basolateral amygdala (AP range: −1.06 to −1.34). 3–6 measurements were taken from each mouse for each brain region. All values were divided by the area of each region and are analyzed as pixels/mm^2 and cells/mm^2, respectively. Tissue was coded so the experimenter was blind to the treatment conditions during analysis.

Stereotaxic Surgery

After 45 self-administration sessions, mice were anesthetized with a cocktail of ketamine (120 mg/kg, i.p.) and xylazine (9 mg/kg, i.p.) and placed into a stereotaxic frame (Kopf Instruments, Tujunga, CA). Mice were implanted with a 26 gauge guide cannula (Plastics One, Roanoke, VA) unilaterally aimed at either the right or the left medial prefrontal cortex (**Experiment 2**; PFC; AP +1.7mm; ML ±0.4mm; DV −1.2mm, from skull surface), bilaterally aimed at the shell of the nucleus accumbens (**Experiment 3**; NAC; AP= −1.42 and ML= +/−0.6 from bregma, DV= −2.9 mm from dura), or bilaterally aimed at the central amygdala (**Experiment 4**; AMY; AP= −1.22 and ML= +/−2.5 from bregma, DV= 3.25 mm from dura). All coordinates were calculated according to the atlas of (Franklin and Paxinos 2001). The guide cannula was secured to the skull with dental cement (Durelon, Butler Schein, Dublin, OH) and a 33 gauge obturator (Plastics One) was inserted after surgery and was moved daily to prevent blockage and scarring. An aversive tasting polish (Bite It©) coated the head-mount and obturator to prevent damage by cage-mates. Mice were monitored until they woke up from anesthesia, after which they were returned to the animal vivarium. Mice recovered from surgery for 1 week before resuming ethanol or sucrose self-administration. A minority of mice (n=12) did not recover from anesthesia or were sick after surgery.

Microinjections & Self-Administration

Sham injections (insertion of the injector and activation of the pump, but no drug delivery) began once stable levels of operant responding were observed after surgery (obtaining >10 reinforcements/session in 3 consecutive sessions). Sham injections were repeated until each mouse responded stably after injection (ca. 3–5 sham injections). Next, dose-effect curves for the MEK inhibitor, SL 327, were conducted for all three brain regions and for each reinforcing solution (6 experimental groups). Vehicle and SL 327 (0.3, 1.0 and 1.7 μg/side) were injected in a counterbalanced design, with a minimum of 3 self-administration sessions separating each test day. Drug solutions were bilaterally infused (0.5 μl/side) over a 4 min period (0.125μl/min/side) using a 1 μl Hamilton syringe connected to a Harvard Apparatus pump (Holliston, MA). A 33 gauge injector (Plastics One) extended 2mm beyond the tip of the guide cannula and remained in place for 1 min after the infusion ended to facilitate drug diffusion and to minimize vertical capillary action along the injection tract. Mice were unrestrained during the infusion and were placed immediately into the operant conditioning chamber for a 1 hr session.

Immediately after the final microinjection, mice were deeply anesthetized with sodium pentobarbital and were intracardially perfused with PBS and PFA. For histological verification, brains were sliced into 50 μm coronal sections and were stained with cresyl violet to visualize the injection site (Figures 1–3, Panels A&B). Subjects with missed cannula placements (n=17), a lost headmount (n=1) and clogged cannula (n=28) were excluded from the final analysis. Unfortunately, a significant minority of subjects in experiments 2 and 3 had clogged cannula due to the requirement that both bilateral cannula be viable and to the slightly sticky nature of the DMSO vehicle.

Microinjections & Locomotor Activity

Open field activity was measured in Plexiglas activity monitor chambers (27.9 cm^2; ENV-510, Med Associates). Two sets of 16 pulse-modulated infrared photobeams recorded X-Y ambulatory movements. The mouse's position in the open field was assessed every 100ms to quantify the distance traveled (cm) throughout the session. Each activity monitor was connected to a computer that compiled all data.

Initially, mice (n=35) were allowed to habituate to the open field apparatus for 2 hrs. One week later, mice with a history of ethanol or sucrose self-administration that did not exhibit stable rates of responding after surgery were given a sham injection prior to being placed in the open field for 1 hr. Vehicle or SL 327 (1.0 and 1.7 μg/side) were injected in a counterbalanced design, with a minimum of 1 week separating each test.

Drugs

All ethanol solutions (v/v) were prepared by diluting 95% ethanol (Pharmco Products Inc.; Brookfield, CT) with water. The MEK inhibitor, SL 327 (α-[Amino[(4-aminophenyl)thio]methylene]-2-(trifluoromethyl)benzeneacetonitrile; Tocris Bioscience; Ellisville, MO) was dissolved in 100% DMSO (dimethlysulfoxide, Mallinckrodt Baker, Inc.; Phillipsburg, NJ) and was diluted to 15% DMSO solution with distilled water.

Statistical Analysis

To assess differences in baseline levels of acquisition of ethanol or sucrose self-administration, a t-test was conducted on the average number of reinforcements obtained in a 1 hr session between ethanol and sucrose self-administering mice for each brain region. For brain region and reinforcing solution, a 2 way repeated measure mixed ANOVA (RM ANOVA) was conducted (DOSE x TIME) on cumulative number of active lever presses and dose consumed (g/kg), respectively. A secondary analysis was conducted to compare the efficacy of SL 327 on the 1st vs 2nd half of the drinking session by collapsing consummatory measures into 2, 30 min bins (0–30 min vs. 31–60 min). A one way RM ANOVA was conducted (DOSE) for this analysis. Locomotor activity was analyzed using one-way RM ANOVA's for each group on total distance traveled in 1hr. Immunohistochemical data was analyzed separately for each subregion using a t-test to compare ethanol and sucrose self-administering groups. When appropriate, all post-hoc analyses were conducted using Dunnett's test with the vehicle condition as the common control. α was set at 0.05 for all comparisons.

Results

Experiment 1: Effect of operant ethanol self-administration on pERK1/2 immunoreactivity

All mice demonstrated stable and comparable levels of ethanol and sucrose self-administration in a 1 hr self-administration session. The average number of reinforcements averaged across 30 drinking sessions did not significantly differ between groups (27.8 ± 3.2 for ethanol and 28.3 ± 4.2 for sucrose self-administering mice) and the average number of reinforcements immediately prior to intracardial perfusion was 30.8 ± 4.9 and 35.4 ± 10.0, respectively. The average dose consumed and BAC at the final operant self-administration session was 1.21 g/kg and 54.9 mg/dl, respectively.

Overall, this experiment revealed that 30 sessions of operant responding for moderate amounts of ethanol induced significant increases in pERK1/2 immunoreactive pixels and cells in most of the regions that were investigated relative to sucrose self-administering mice. Specifically, in the medial prefrontal cortex, mice with a history of operant ethanol self-administration had significantly increased levels of immunoreactive cells ($t(11) = 2.140$, $p=0.0278$) and pixels for pERK1/2 ($t(11) = 2.646$, $p=0.0114$, Figure 1A) as compared to sucrose self-administering mice. Levels of pERK1/2 immunoreactive cells and pixels were significantly higher in both the core (cells: $t(13) = 1.935$, $p=0.038$; pixels: $t(13) = 1.928$, $p=0.038$, Figure 1B) and shell (cells: $t(14) = 1.74$, $p=0.051$; pixels: $t(14) = 2.035$, $p=0.030$, Figure 1B) of the nucleus accumbens in ethanol but not sucrose self-administering mice. In the amygdala, there was a differential effect between the two sub-regions that were analyzed. Namely, an increase in pERK1/2 immunoreactive pixels and cells was found in the CeA (cells: $t(14) = 2.976$, $p=0.005$; pixels: $t(14) = 2.797$, $p=0.007$, Figure 1C) but not in the BLA.

Experiment 2: ERK1/2 inhibition in the prefrontal cortex

All mice demonstrated stable and comparable levels of ethanol and sucrose self-administration in a 1 hr self-administration session. Across all experiments, the drinking

troughs were empty at the end of the session. The average number of reinforcements averaged across 45 drinking sessions did not significantly differ between groups (23.0 ± 2.2 for ethanol+sucrose and 27.2 ± 2.9 for sucrose self-administering mice). Histological analysis showed that n=32 mice received injections into the PFC (Figure 2A–B)

Intra-PFC microinjection of SL 327 produced a robust increase in ethanol self-administration but a decrease in sucrose self-administration. Both drug effects were independent from concomitant locomotor-impairing effects and the pharmacological effect of SL 327 did not differ according to the side the cannula was placed in. Specifically, there was a significant main effect of time (Figure 2C; $F_{(11,198)} = 31.4$, $p < 0.001$) and of SL 327 dose (Figure 2C; $F_{(3,198)} = 4.0$, $p=0.023$) on the number of active lever presses in ethanol self-administering mice. Post-hoc tests revealed that the highest dose of SL 327 (1.7μg) significantly increased active lever responding. Similarly, there was a significant main effect of time ($F_{(11,198)} = 31.6$, $p < 0.001$) and of SL 327 dose ($F_{(3,198)} = 3.9$, $p=0.026$) on the total dose consumed (g/kg) during the session. Post-hoc tests revealed that a higher g/kg was obtained after intra-PFC administration of 1.7μg of SL 327 as compared to vehicle (0.92 ± .11 vs. 0.69 ± .11 g/kg/hr). Responding on the inactive lever was not affected by treatment condition. Given the significant main effect of time and to more specifically address the temporal effect of SL 327 on operant responding, data were collapsed into two 30 min bins and were analyzed independently (Figure 2D–E). A significant main effect of SL 327 dose was found within the first 30 min after infusion of the drug ($F_{(3,21)} = 3.7$, $p=0.028$; Figure 2D). A post-hoc test revealed that this main effect was due to a significant increase in operant responding for ethanol after infusion of 1.7μg SL 327. This drug effect was no longer present in the 2nd half of the self-administration session (Figure 2E).

In sucrose self-administering mice, there was a significant main effect of time (Figure 2F; $F_{(11,198)} = 34.4$, $p < 0.001$), as well as a significant interaction between time and SL 327 dose (Figure 2F; $F_{(33,198)} = 2.66$, $p < 0.001$) on the number of active lever presses obtained. Post-hoc tests revealed that intra-PFC administration of 0.3 and 1.7μg of SL 327 significantly decreased active lever responding by minute 45 of the session and that all doses significantly decreased operant responding for sucrose during the last 15 min of the drinking session. Responding on the inactive lever was not affected by treatment condition. When data were collapsed into two 30 min bins and analyzed independently, a one way RM ANOVA revealed a significant main effect of SL 327 dose in the 2nd half of the session, during min 31–60 ($F_{(3,21)} = 3.7$, $p=0.028$; Figure 2H). A post-hoc test revealed that this main effect was due to a significant decrease in operant responding for sucrose after infusion of 1.7μg SL 327. In contrast to ethanol self-administering mice, this drug effect was not present in the 1nd half of the self-administration session (Figure 2G).

Mice that did not lever press consistently after surgery or after sham injections (n=15) were used to test whether SL327 altered spontaneous locomotor activity. They were first habituated to the locomotor chambers and subsequently, placed into the chambers for 1 hr sessions following a microinjection of vehicle or SL 327. During the 2 hr habituation session, mice moved, on average, 20,824 ± 1024 cm/2hrs. Distance traveled after vehicle injection did not differ between groups and intra-PFC microinjection of SL 327 did not affect spontaneous locomotor activity (Table 1).

Experiment 3: ERK1/2 inhibition in the nucleus accumbens

All mice demonstrated stable and comparable levels of ethanol and sucrose self-administration in a 1 hr self-administration session. The average number of reinforcements averaged across 45 drinking sessions did not significantly differ between groups (29.1 ± 3.2 for ethanol and 33.8 ± 4.9 for sucrose self-administering mice). Histological analysis showed that n=22 mice received injections predominantly into the shell region of the NAC (Figure 3A–B).

Unlike results observed in the PFC, SL 327 infusion into the NAC did not significantly alter operant responding for ethanol throughout the 1 hr session (Figure 3C–E). The average dose consumed after intra-NAC microinjection of vehicle was 0.73 ± .15 g/kg/hr and was insensitive to intra-NAC microinjection of SL 327. Likewise, responding on the inactive lever was not affected by treatment condition.

In contrast, sucrose self-administration showed a significant main effect of time (Figure 3F; $F_{(11,176)} = 25.8$, $p < 0.001$), dose (Figure 3F; $F_{(11,176)} = 12.3$, $p < 0.001$), and a significant interaction of TIME x DOSE (Figure 3F; $F_{(22,176)} = 7.5$, $p < 0.001$) on the number of active lever presses. Post-hoc tests revealed that intra-PFC administration of both doses of SL 327 (1.0 μg and 1.7μg) significantly decreased active lever responding. Moreover, the reduction of responding elicited by intra-NAC microinjection of 1.7μg of SL 327 emerged 15 min into the self-administration session and the effects of 1.0μg of SL 327 emerged by 30 min (Figure 3G–H). Likewise, inactive lever responding was also reduced by intra-NAC injection of SL 327. For this behavior, there was a significant main effect of time ($F_{(11,176)} = 10.3$, $p < 0.001$) and a significant interaction of TIME x DOSE ($F_{(22,176)} = 3.7$, $p < 0.001$). Post-hoc tests revealed that intra-PFC administration of 1.0 μg and 1.7 μg of SL 327 significantly decreased active lever responding and that this effect emerged 50 min into the self-administration session. When data were collapsed into two 30 min bins and analyzed independently, a one way RM ANOVA revealed a significant main effect of SL 327 dose during min 0–30 ($F_{(2,12)} = 6.3$, $p=0.013$; Figure 3H) and during min 31–60 ($F_{(2,12)} = 7.6$, $p=0.007$; Figure 3H). For both time bins, this effect was due to a significant reduction in operant responding for sucrose after infusion of both doses of SL 327.

Mice that did not lever press consistently after surgery or after sham injections (n=10) were used to test whether SL327 altered spontaneous locomotor activity. They were first habituated to the locomotor chambers and subsequently, placed into the chambers for 1 hr sessions following a microinjection of vehicle or SL 327. During the 2 hr habituation session, mice moved, on average, 11,776 ± 1557 cm/2hrs. Activity levels after vehicle injection did not differ between ethanol and sucrose groups and were unaffected by SL 327 administration (Table 1).

Experiment 4: ERK1/2 inhibition in the amygdala

All mice demonstrated stable and comparable levels of ethanol and sucrose self-administration in a 1 hr self-administration session. The average number of reinforcements averaged across 45 drinking sessions did not significantly differ between groups (26.6 ± 4.7 for ethanol and 18.3 ± 2.5 for sucrose self-administering mice). Histological analysis

showed that the majority of mice received injections aimed at the central nucleus of the amygdala though some injections occurred in the basolateral amygdala (n=22; Figure 4A–B).

Although there was a trend for increased self-administration after drug microinjection, overall, intra-amygdala microinjection of SL 327 did not significantly modulate either ethanol or sucrose self-administration. Specifically, there was a significant main effect of time, but not of dose, on the number of active lever presses (Figure 4C–E; $F_{(11,132)} = 47.4$, $p < 0.001$) in ethanol self-administering mice. The average dose consumed (g/kg) after intra-amygdala microinjection of vehicle was 0.63 ± .16 g/kg/hr and was not affected by intra-amygdala microinjection of SL 327. Additionally, responding on the inactive lever was not affected by treatment condition.

Likewise, sucrose self-administering mice also showed a significant main effect of time (Figure 4F–H; $F_{(11,198)} = 32.0$, $p < 0.001$), but not of dose, on the number of cumulative active lever presses obtained. This effect was selective to the active lever as inactive responding was unaffected by treatment condition.

Mice that did not lever press consistently after surgery or after sham injections (n=10) were used to test whether SL327 altered spontaneous locomotor activity. They were first habituated to the locomotor chambers and subsequently, placed into the chambers for 1 hr sessions following a microinjection of vehicle or SL 327. During the 2 hr habituation session, mice moved, on average, 19,723 ± 953 cm/2hrs. Distance traveled after vehicle injection did not differ between groups and were unaffected by SL 327 microinjection (Table 1).

Discussion

Repeated drug use produces neuroadaptive changes in molecular and cell signaling pathways that, in turn, lead to long-lasting neural and behavioral pathologies that characterize addiction (Nestler 2001). Among these cellular systems, the activity-dependent MAPK pathway is crucial for the transduction of neuronal activity into immediate or enduring changes in neural plasticity and is critical in addiction (Girault et al. 2007; Thomas and Huganir 2004). The present work extends and refines the functional role of ERK/MAPK in the direct regulation of the positive reinforcing effects of ethanol (Faccidomo et al. 2009). Results from this study first demonstrate that the positive reinforcing effects of ethanol are associated with increased activation of ERK1/2 in the PFC, NAC and AMY. These immunohistochemical data confirm prior studies showing that acute ethanol administration (Agoglia et al. 2015; Ibba et al. 2009; Rosas et al. 2014; Spanos et al. 2012; Valjent et al. 2004) and chronic ethanol intake (Pandey et al. 2008; Zhu et al. 2013) can up- or down-regulate levels of pERK1/2 in a brain region-dependent manner. The present findings extend this research by showing that operant self-administration of moderate doses of ethanol robustly increases pERK1/2 immumoreactivity above what is engendered by the non-drug reinforcer, sucrose. Here we also sought to determine if ERK activity in the PFC, NAC, or AMY mechanistically regulates operant alcohol self-administration. Results show that site-specific infusion of the MEK/ERK inhibitor SL 327 potentiated ethanol self-administration

only when this drug was infused into the PFC. These data are the first direct evidence to show that ERK1/2 activity in the prefrontal cortex is functionally regulates the positive reinforcing properties of ethanol.

Although the specific mechanisms by which self-administered ethanol increased pERK1/2 immunoreactivity in the PFC, NAC, and AMY remain to be determined, several well-characterized biochemical effects of ethanol may contribute to this process. For instance, ERK1/2 phosphorylation requires the coordinated activation of dopamine D1-like and NMDA receptors (Shiflett and Balleine 2011). Ethanol increases dopamine levels in the NAC (Di Chiara and Imperato 1986; Olive et al. 2000) and PFC (Schier et al. 2013), and elevates glutamate levels in the NAC (Ding et al. 2013; Griffin et al. 2014; Szumlinski et al. 2007) and AMY (Roberto et al. 2004). Moreover, ethanol activates ERK1/2 via a D1-dependent mechanism in NAC and AMY (Ibba et al. 2009). Thus, it is plausible that self-administered ethanol produces a coordinated increase in dopamine and glutamate levels in mesocorticolimbic brain regions that leads to downstream ERK1/2 activation.

A highly significant finding from this study is that ERK1/2 phosphorylation in the PFC is a molecular target of self-administered ethanol that, in turn, mechanistically regulates the positive reinforcing effects of the drug. We previously discovered that the PFC actively regulates behavioral plasticity in addiction by modulating the positive reinforcing effects of ethanol (Hodge et al. 1996) and that ethanol increases ERK1/2 phosphorylation in the PFC (Spanos et al. 2012). In this study, pharmacological inhibition of ERK1/2 activity in the PFC via local infusion of the MEK inhibitor SL 327 produced a significant increase in ethanol reinforced responding in the absence of non-specific motor effects, which is consistent with prior evidence indicating that systemic ERK1/2 inhibition produces the same behavioral effect (Faccidomo et al. 2009). Results also showed that operant self-administration of relatively low doses of ethanol (1.21 g/kg; BAC = 54.9 mg/dl) significantly increased pERK1/2 phosphorylation in the PFC. Thus, we hypothesize that SL 327 infusion in the PFC increased the reinforcing effects of ethanol because it inhibited a critical molecular consequence of ethanol-self administration – e.g., increased pERK1/2 activity. Thus, the increased rate of ethanol self-administration may reflect a form of behavioral compensation to overcome this pharmacological “antagonism” of ethanol-induced ERK1/2 activity by MEK inhibition in the PFC. Similarly, inhibition of ERK1/2 signaling in the PFC may have escalated ethanol self-administration via altered function of PFC-linked neural circuits, affecting downstream components of the mesocorticolimbic system. For example, ERK1/2 inhibition in PFC may inhibit excitatory glutamatergic projections that terminate in the NAC (Berendse et al. 1992) where glutamate transmission promotes increased alcohol drinking (Griffin et al. 2014; Kapasova and Szumlinski 2008) and is required for the positive reinforcing (Besheer et al. 2010) and discriminative stimulus (Besheer et al. 2009) effects of ethanol. Moreover, dysregulated PFC to NAC glutamate transmission may drive increased motivation to seek a variety of drugs (Kalivas et al. 2005). Overall, these findings indicate that ERK1/2 signaling in the PFC modulates the positive reinforcing properties of ethanol, which suggests that this pathway may influence the development of addiction (Wise and Koob 2014).

Unlike in the PFC, pharmacological inhibition of ERK1/2 activity in the NAC and AMY did not affect ethanol reinforced responding. However, responding for sucrose was significantly blunted by both doses of SL 327, beginning 15 min after microinfusion into the NAC and persisting throughout the self-administration session. This result supports the hypothesis that under these experimental conditions, inhibition of the ERK1/2 pathway in the NAC appears to mediate processes involved in regulating the motivation for non-drug rewards. The NAC is activated in response to both appetitive and drug stimuli that are rewarding and many labs have indeed demonstrated that the NAC dynamically regulates the motivation for multiple types of goal-directed behaviors (Bassareo et al. 2011; Di Chiara and Bassareo 2007). It is also evident that sub-populations of neurons within this region distinctly respond to different types of reinforcers (i.e. ethanol vs. water vs. sucrose; (Bassareo et al. 2011; Carelli et al. 2000; Carelli and Wondolowski 2006; Robinson and Carelli 2008; Roop et al. 2002). Although we were unable to differentially target distinct populations of neurons, our results suggest that NAC neurons that respond to non-drug rewards such as sucrose may utilize ERK1/2 cell signaling in the dynamic regulation of motivated behavior for non-drug rewards.

Food restriction can enhance both the behavioral and central reinforcing effects of drugs of abuse by increasing mesolimbic DA signaling and consequently, the salience of the rewarding substance (Cabeza de Vaca and Carr 1998; Carr 2002; Carr et al. 2001; Carroll et al. 1979). ERK1/2 is a downstream molecular target of D1 receptors in the NAC and this kinase is elevated in food-restricted rats, both at baseline and after injection with a D1 receptor agonist or cocaine (Haberny et al. 2004; Liu et al. 2011). Importantly, acute ethanol-induced increase in pERK1/2 can be blocked by pretreatment with a D1 receptor antagonist (Ibba et al. 2009) and pharmacological inhibition of ERK1/2 in the nucleus accumbens by SL 327 does not alter baseline self-stimulation threshold and is not rewarding (Carr et al. 2009). Moreover, intra-VTA infusion of the MEK inhibitor U0126 does not alter ethanol–reinforced responding in highly trained rats suggesting that ERK regulation along the mesolimbic dopaminergic pathway is not directly regulating ethanol reinforcement (Carnicella et al. 2008). Taken together with our findings, these data suggest that the D1-ERK intracellular signaling cascade in the nucleus accumbens is resistant to manipulation by SL 327 but sensitive to pharmacological blockade of postsynaptic dopamine receptors. Thus, a possible explanation for the absence of an effect of intra-NAC ERK inhibition on ethanol reinforcment is that once the association between the reinforcer and the behavior is learned, accumbal DA is no longer required for the behavior to occur and the D1-ERK cascade in the accumbens is no longer engaged. A second possible consequence to repeated alcohol exposure is tolerance to the ethanol-induced increase in ERK1/2 activation. However, Spanos et al. (2012) and Agoglia et al. (2015) show that acute ethanol increases pERK1/2 immunoreactivity in the NAC and the data from Experiment 4 show a similar effect after chronic self-administration of sweetened ethanol. Thus, under the current conditions, tolerance to ethanol-induced ERK1/2 activation in this region was not observed. An interesting future experiment would be to inhibit ERK1/2 in this pathway during the early acquisition of operant responding for ethanol in untrained animals to determine if this signaling pathway in the nucleus accumbens might be more influential during the

development of the learned relationship between the behavioral response (lever press) and the reinforcer (ethanol).

Notably, while all groups had similar rates of responding during the acquisition phase of training (ie: the average number of reinforcements for ethanol and sucrose mice did not differ), rates of responding declined after intra-AMY infusion of vehicle in both groups. It is likely that these mice were sensitive to the extra handling required for these microinjection studies given that 2 separate injectors were bilaterally inserted into this region. Importantly, despite the decrease in response rate in the amygdala cohorts, there was not a significant difference between ethanol- and sucrose-reinforced responses after vehicle microinjection. Thus, it is unlikely that the decrease in response rate impacted the effect of SL 327 on responding since these were within-subject experiments. Given the absence of difference in response rates within experiments between ethanol and sucrose mice, it also does not seem likely that caloric differences in the consumed reinforcing solution between ethanol and sucrose groups (approximately 0.172 and 0.022 calories, respectively) accounted for the differences in drug effects. Their food contains 4.1 cal/gram and each mouse eats on average 20 cal/day. Thus, the number of calories that are contributing to the diet from both the 2% sucrose and 2% sucrose/9% ethanol solution is <1% of total daily intake and as such, it is highly unlikely that these caloric differences in the reinforcing solutions are sufficient to alter the pharmacological effects of SL 327.

The ERK/MAPK pathway in the central nucleus of the amygdala (CeA) is activated in response to acute ethanol injection and home cage 24-h and binge 4-h self-administration, (Ibba et al. 2009; Spanos et al. 2012; Thorsell et al. 2013). Likewise, we found that 30 days of operant ethanol self-administration significantly increased pERK1/2 IHC in the CeA but not BLA (Figure 1C). Together, these data show that ethanol-induced activation of pERK1/2 in the CeA is a strikingly robust and consistent molecular response to ethanol that occurs via multiple routes, schedules and methods of ethanol self-administration. Thus, the pharmacological results from experiment 3 are somewhat difficult to interpret as ERK1/2 inhibition in the amygdala did not modulate ethanol or sucrose reinforced responding. Others have shown that dampening activity in the central and medial amygdala promotes home cage drinking (Pandey et al. 2006), perhaps due to enhancement of an anxiogenic state. However, this effect is restricted to the medial and central amygdala where basal ERK1/2 levels are elevated as a consequence of ethanol drinking. Given the physical limitations of our microinjections, it was not possible in this study to selectively inhibit ERK1/2 in the central and medial vs. the basolateral amygdala although we would predict that selectively inhibition of ERK1/2 in only CeA neurons would produce an increase in ethanol self-administration. An alternative explanation is that under these conditions, ERK1/2 activity in the amygdala is less involved in modulating reinforcement and more involved in regulating cue-induced reinstatement of drug-seeking, or relapse-like behavior, rather than drug self-administration. For instance, pERK1/2 immunoreactivity in the BLA and CeA are upregulated in abstinent animals after exposure to ethanol and cocaine-associated cues suggesting that these sub-nuclei are dynamically activated by salient drug cues and may be more involved in modulating drug-seeking rather than drug-taking (Lu et al. 2005; Radwanska et al. 2007; Schroeder et al. 2008). Moreover, pretreatment with the glutamatergic antagonists, MPEP and AP-5, as well as the MEK inhibitor, U0126, blocks

both the cue-associated activation of pERK1/2 and responding on the active lever during a reinstatement session, indicating that cue-induced reinstatement may be mediated by both glutamate and ERK1/2 activity. Thus, the present study may have failed to find evidence for functional involvement of the ERK1/2 activity in the amygdala in ethanol-reinforced responding because ERK1/2 in this region may regulate cue-induced drug-seeking, or relapse-like, behavior rather than primary reinforcement or drug-taking behavior.

Collectively, the most salient finding from these studies is that a history of ethanol self-administration induces an increase in ERK1/2 phosphorylation in multiple reward-relevant brain regions yet inhibition of ERK1/2 activity only in the PFC preferentially increases operant responding for ethanol (Table 2). Given that acute ethanol leads to a transient increase in pERK1/2 expression in reward-related brain regions, it is likely that pharmacological blockade of ERK1/2 phosphorylation is inhibiting ethanol-induced ERK1/2 phosphorylation which is promoting escalated operant responding and ethanol self-administration in a compensatory manner. Though we cannot definitively resolve why a similar functional relationship did not emerge in the amygdala and nucleus accumbens, we can speculate that the lack of subnuclei selectivity with our injections may have masked a functional effect of the inhibitor on reinforcing properties of ethanol in these regions. In conclusion, it is evident that the PFC is the key region that underlies ERK's modulation of the positive reinforcing effects of ethanol self-administration in a mouse model of ethanol self-administration.

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References

Adams JP, Sweatt JD. Molecular psychology: roles for the ERK MAP kinase cascade in memory. Annu Rev Pharmacol Toxicol. 2002; 42:135–63. [PubMed: 11807168]

Agoglia AE, Sharko AC, Psilos KE, Holstein SE, Reid GT, Hodge CW. Alcohol Alters the Activation of ERK1/2, a Functional Regulator of Binge Alcohol Drinking in Adult C57BL/6J Mice. Alcohol Clin Exp Res. 2015; 39:463–75. [PubMed: 25703719]

Arolfo MP, Overstreet DH, Yao L, Fan P, Lawrence AJ, Tao G, Keung WM, Vallee BL, Olive MF, Gass JT, Rubin E, Anni H, Hodge CW, Besheer J, Zablocki J, Leung K, Blackburn BK, Lange LG, Diamond I. Suppression of heavy drinking and alcohol seeking by a selective ALDH-2 inhibitor. Alcohol Clin Exp Res. 2009; 33:1935–44. [PubMed: 19673742]

Bassareo V, Musio P, Di Chiara G. Reciprocal responsiveness of nucleus accumbens shell and core dopamine to food- and drug-conditioned stimuli. Psychopharmacology (Berl). 2011; 214:687–697. [PubMed: 21110007]

Berendse HW, Galis-de Graaf Y, Groenewegen HJ. Topographical organization and relationship with ventral striatal compartments of prefrontal corticostriatal projections in the rat. The Journal of comparative neurology. 1992; 316:314–47. [PubMed: 1577988]

Besheer J, Fisher KR, Cannady R, Grondin JJ, Hodge CW. Intra-amygdala inhibition of ERK(1/2) potentiates the discriminative stimulus effects of alcohol. Behav Brain Res. 2012; 228:398–405. [PubMed: 22209853]

Besheer J, Grondin JJ, Cannady R, Sharko AC, Faccidomo S, Hodge CW. Metabotropic glutamate receptor 5 activity in the nucleus accumbens is required for the maintenance of ethanol self-administration in a rat genetic model of high alcohol intake. Biol Psychiatry. 2010; 67:812–22. [PubMed: 19897175]

Besheer J, Grondin JJ, Salling MC, Spanos M, Stevenson RA, Hodge CW. Interoceptive effects of alcohol require mGlu5 receptor activity in the nucleus accumbens. J Neurosci. 2009; 29:9582–91. [PubMed: 19641121]

Besnard A, Laroche S, Caboche J. Comparative dynamics of MAPK/ERK signalling components and immediate early genes in the hippocampus and amygdala following contextual fear conditioning and retrieval. Brain Struct Funct. 2014; 219:415–30. [PubMed: 23389809]

Cabeza de Vaca S, Carr KD. Food restriction enhances the central rewarding effect of abused drugs. J Neurosci. 1998; 18:7502–10. [PubMed: 9736668]

Cannady R, Fisher KR, Durant B, Besheer J, Hodge CW. Enhanced AMPA receptor activity increases operant alcohol self-administration and cue-induced reinstatement. Addict Biol. 2013; 18:54–65. [PubMed: 23126443]

Carelli RM, Ijames SG, Crumling AJ. Evidence that separate neural circuits in the nucleus accumbens encode cocaine versus “natural” (water and food) reward. J Neurosci. 2000; 20:4255–66. [PubMed: 10818162]

Carelli RM, Wondolowski J. Anatomic distribution of reinforcer selective cell firing in the core and shell of the nucleus accumbens. Synapse. 2006; 59:69–73. [PubMed: 16270303]

Carnicella S, Kharazia V, Jeanblanc J, Janak PH, Ron D. GDNF is a fast-acting potent inhibitor of alcohol consumption and relapse. Proc Natl Acad Sci U S A. 2008; 105:8114–9. [PubMed: 18541917]

Carr KD. Augmentation of drug reward by chronic food restriction: behavioral evidence and underlying mechanisms. Physiol Behav. 2002; 76:353–64. [PubMed: 12117572]

Carr KD, de Vaca SC, Sun Y, Chau LS, Pan Y, Dela Cruz J. Effects of the MEK inhibitor, SL-327, on rewarding, motor- and cellular-activating effects of D-amphetamine and SKF-82958, and their augmentation by food restriction in rat. Psychopharmacology (Berl). 2009; 201:495–506. [PubMed: 18766328]

Carr KD, Kim GY, Cabeza de Vaca S. Rewarding and locomotor-activating effects of direct dopamine receptor agonists are augmented by chronic food restriction in rats. Psychopharmacology (Berl). 2001; 154:420–8. [PubMed: 11349397]

Carroll ME, France CP, Meisch RA. Food deprivation increases oral and intravenous drug intake in rats. Science. 1979; 205:319–21. [PubMed: 36665]

Chandler LJ, Sutton G. Acute ethanol inhibits extracellular signal-regulated kinase, protein kinase B, and adenosine 3′:5′-cyclic monophosphate response element binding protein activity in an age- and brain region-specific manner. Alcohol Clin Exp Res. 2005; 29:672–82. [PubMed: 15834234]

Clapp P. Current progress in pharmacologic treatment strategies for alcohol dependence. Expert Rev Clin Pharmacol. 2012; 5:427–35. [PubMed: 22943122]

Committee for the Update of the Guide for the Care and Use of Laboratory Animals IfLAR. Guide for the Care and Use of Laboratory Animals Guide for the Care and Use of Laboratory Animals (The National Academies Collection: Reports funded by National Institutes of Health). National Academies Press; Washington (DC): 2011. p. 220

Cui C, Noronha A, Morikawa H, Alvarez VA, Stuber GD, Szumlinski KK, Kash TL, Roberto M, Wilcox MV. New insights on neurobiological mechanisms underlying alcohol addiction. Neuropharmacology. 2013; 67:223–32. [PubMed: 23159531]

Cui SZ, Wang SJ, Li J, Xie GQ, Zhou R, Chen L, Yuan XR. Alteration of synaptic plasticity in rat dorsal striatum induced by chronic ethanol intake and withdrawal via ERK pathway. Acta Pharmacol Sin. 2011; 32:175–81. [PubMed: 21293469]

Davis S, Vanhoutte P, Pages C, Caboche J, Laroche S. The MAPK/ERK cascade targets both Elk-1 and cAMP response element-binding protein to control long-term potentiation-dependent gene expression in the dentate gyrus in vivo. J Neurosci. 2000; 20:4563–72. [PubMed: 10844026]

Di Chiara G, Imperato A. Preferential stimulation of dopamine release in the nucleus accumbens by opiates, alcohol, and barbiturates: studies with transcerebral dialysis in freely moving rats. Ann N Y Acad Sci. 1986; 473:367–81. [PubMed: 3467628]

Ding ZM, Rodd ZA, Engleman EA, Bailey JA, Lahiri DK, McBride WJ. Alcohol drinking and deprivation alter basal extracellular glutamate concentrations and clearance in the mesolimbic system of alcohol-preferring (P) rats. Addict Biol. 2013; 18:297–306. [PubMed: 23240885]

Faccidomo S, Besheer J, Stanford PC, Hodge CW. Increased operant responding for ethanol in male C57BL/6J mice: specific regulation by the ERK(1/2), but not JNK, MAP kinase pathway. Psychopharmacology (Berl). 2009

Franklin, KBJ.; Paxinos, G. The Mouse Brain in Sterotaxic Coordinates. 2. Academic Press; New York: 2001.

Girault JA, Valjent E, Caboche J, Herve D. ERK2: a logical AND gate critical for drug-induced plasticity? Curr Opin Pharmacol. 2007; 7:77–85. [PubMed: 17085074]

Goulding SP, Obara I, Lominac KD, Gould AT, Miller BW, Klugmann M, Szumlinski KK. Accumbens Homer2-mediated signaling: a factor contributing to mouse strain differences in alcohol drinking? Genes Brain Behav. 2011; 10:111–26. [PubMed: 20807241]

Grant BF, Dawson DA, Stinson FS, Chou SP, Dufour MC, Pickering RP. The 12-month prevalence and trends in DSM-IV alcohol abuse and dependence: United States, 1991–1992 and 2001–2002. Drug Alcohol Depend. 2004; 74:223–34. [PubMed: 15194200]

Grant KA, Samson HH. Oral self administration of ethanol in free feeding rats. Alcohol. 1985; 2:317–21. [PubMed: 4040380]

Griffin WC 3rd, Haun HL, Hazelbaker CL, Ramachandra VS, Becker HC. Increased extracellular glutamate in the nucleus accumbens promotes excessive ethanol drinking in ethanol dependent mice. Neuropsychopharmacology. 2014; 39:707–17. [PubMed: 24067300]

Groblewski PA, Franken FH, Cunningham CL. Inhibition of extracellular signal-regulated kinase (ERK) activity with SL327 does not prevent acquisition, expression, and extinction of ethanol-seeking behavior in mice. Behav Brain Res. 2011; 217:399–407. [PubMed: 21074569]

Guo ML, Xue B, Jin DZ, Mao LM, Wang JQ. Interactions and phosphorylation of postsynaptic density 93 (PSD-93) by extracellular signal-regulated kinase (ERK). Brain Res. 2012; 1465:18–25. [PubMed: 22618309]

Haberny SL, Berman Y, Meller E, Carr KD. Chronic food restriction increases D-1 dopamine receptor agonist-induced phosphorylation of extracellular signal-regulated kinase 1/2 and cyclic AMP response element-binding protein in caudate-putamen and nucleus accumbens. Neuroscience. 2004; 125:289–98. [PubMed: 15051167]

Hasin DS, Stinson FS, Ogburn E, Grant BF. Prevalence, correlates, disability, and comorbidity of DSM-IV alcohol abuse and dependence in the United States: results from the National Epidemiologic Survey on Alcohol and Related Conditions. Arch Gen Psychiatry. 2007; 64:830–42. [PubMed: 17606817]

Hodge CW, Chappelle AM, Samson HH. Dopamine receptors in the medial prefrontal cortex influence ethanol and sucrose-reinforced responding. Alcohol Clin Exp Res. 1996; 20:1631–8. [PubMed: 8986215]

Hodge CW, Samson HH, Lewis RS, Erickson HL. Specific decreases in ethanol- but not water-reinforced responding produced by the 5-HT3 antagonist ICS 205-930. Alcohol. 1993; 10:191–6. [PubMed: 8507386]

Ibba F, Vinci S, Spiga S, Peana AT, Assaretti AR, Spina L, Longoni R, Acquas E. Ethanol-induced extracellular signal regulated kinase: role of dopamine D1 receptors. Alcohol Clin Exp Res. 2009; 33:858–67. [PubMed: 19320634]

Jeanblanc J, Logrip ML, Janak PH, Ron D. BDNF-mediated regulation of ethanol consumption requires the activation of the MAP kinase pathway and protein synthesis. Eur J Neurosci. 2013; 37:607–12. [PubMed: 23189980]

Kalivas PW, Volkow N, Seamans J. Unmanageable motivation in addiction: a pathology in prefrontal-accumbens glutamate transmission. Neuron. 2005; 45:647–50. [PubMed: 15748840]

Kapasova Z, Szumlinski KK. Strain differences in alcohol-induced neurochemical plasticity: a role for accumbens glutamate in alcohol intake. Alcohol Clin Exp Res. 2008; 32:617–31. [PubMed: 18341649]

Liu S, Zheng D, Peng XX, Cabeza de Vaca S, Carr KD. Enhanced cocaine-conditioned place preference and associated brain regional levels of BDNF, p-ERK1/2 and p-Ser845-GluA1 in food-restricted rats. Brain Res. 2011; 1400:31–41. [PubMed: 21640333]

Lu L, Hope BT, Dempsey J, Liu SY, Bossert JM, Shaham Y. Central amygdala ERK signaling pathway is critical to incubation of cocaine craving. Nat Neurosci. 2005; 8:212–9. [PubMed: 15657599]

Mann K. Pharmacotherapy of alcohol dependence: a review of the clinical data. CNS Drugs. 2004; 18:485–504. [PubMed: 15182219]

Meisch RA, Thompson T. Ethanol as a reinforcer: effects of fixed-ratio size and food deprivation. Psychopharmacologia. 1973; 28:171–83. [PubMed: 4694625]

Nestler EJ. Molecular basis of long-term plasticity underlying addiction. Nat Rev Neurosci. 2001; 2:119–28. [PubMed: 11252991]

Olive MF, Mehmert KK, Messing RO, Hodge CW. Reduced operant ethanol self-administration and in vivo mesolimbic dopamine responses to ethanol in PKCepsilon-deficient mice. Eur J Neurosci. 2000; 12:4131–40. [PubMed: 11069609]

Pandey SC, Zhang H, Roy A, Misra K. Central and medial amygdaloid brain-derived neurotrophic factor signaling plays a critical role in alcohol-drinking and anxiety-like behaviors. J Neurosci. 2006; 26:8320–31. [PubMed: 16899727]

Pandey SC, Zhang H, Ugale R, Prakash A, Xu T, Misra K. Effector immediate-early gene arc in the amygdala plays a critical role in alcoholism. J Neurosci. 2008; 28:2589–600. [PubMed: 18322102]

Qin Y, Zhu Y, Baumgart JP, Stornetta RL, Seidenman K, Mack V, van Aelst L, Zhu JJ. State-dependent Ras signaling and AMPA receptor trafficking. Genes Dev. 2005; 19:2000–15. [PubMed: 16107614]

Radwanska K, Wrobel E, Korkosz A, Rogowski A, Kostowski W, Bienkowski P, Kaczmarek L. Alcohol Relapse Induced by Discrete Cues Activates Components of AP-1 Transcription Factor and ERK Pathway in the Rat Basolateral and Central Amygdala. Neuropsychopharmacology. 2007

Rimondini R, Arlinde C, Sommer W, Heilig M. Long-lasting increase in voluntary ethanol consumption and transcriptional regulation in the rat brain after intermittent exposure to alcohol. Faseb J. 2002; 16:27–35. [PubMed: 11772933]

Roberto M, Schweitzer P, Madamba SG, Stouffer DG, Parsons LH, Siggins GR. Acute and chronic ethanol alter glutamatergic transmission in rat central amygdala: an in vitro and in vivo analysis. J Neurosci. 2004; 24:1594–603. [PubMed: 14973247]

Robinson DL, Carelli RM. Distinct subsets of nucleus accumbens neurons encode operant responding for ethanol versus water. European Journal of Neuroscience. 2008; 28:1887–1894. [PubMed: 18973602]

Roop RG, Hollander JA, Carelli RM. Accumbens activity during a multiple schedule for water and sucrose reinforcement in rats. Synapse. 2002; 43:223–6. [PubMed: 11835516]

Rosas M, Zaru A, Sabariego M, Giugliano V, Carboni E, Colombo G, Acquas E. Differential sensitivity of ethanol-elicited ERK phosphorylation in nucleus accumbens of Sardinian alcohol-preferring and -non preferring rats. Alcohol. 2014; 48:471–6. [PubMed: 24877898]

Samson HH, Pfeffer AO, Tolliver GA. Oral ethanol self-administration in rats: models of alcohol-seeking behavior. Alcohol Clin Exp Res. 1988; 12:591–8. [PubMed: 3067600]

Sanna PP, Simpson C, Lutjens R, Koob G. ERK regulation in chronic ethanol exposure and withdrawal. Brain Res. 2002; 948:186–91. [PubMed: 12383974]

Schier CJ, Dilly GA, Gonzales RA. Intravenous ethanol increases extracellular dopamine in the medial prefrontal cortex of the Long-Evans rat. Alcohol Clin Exp Res. 2013; 37:740–7. [PubMed: 23421849]

Schroeder JP, Olive F, Koenig H, Hodge CW. Intra-amygdala infusion of the NPY Y1 receptor antagonist BIBP 3226 attenuates operant ethanol self-administration. Alcohol Clin Exp Res. 2003; 27:1884–91. [PubMed: 14691375]

Schroeder JP, Spanos M, Stevenson JR, Besheer J, Salling M, Hodge CW. Cue-induced reinstatement of alcohol-seeking behavior is associated with increased ERK1/2 phosphorylation in specific limbic brain regions: blockade by the mGluR5 antagonist MPEP. Neuropharmacology. 2008; 55:546–54. [PubMed: 18619984]

Self DW. Regulation of drug-taking and -seeking behaviors by neuroadaptations in the mesolimbic dopamine system. Neuropharmacology. 2004; 47(Suppl 1):242–55. [PubMed: 15464141]

Shiflett MW, Balleine BW. Contributions of ERK signaling in the striatum to instrumental learning and performance. Behav Brain Res. 2011; 218:240–7. [PubMed: 21147168]

Spanos M, Besheer J, Hodge CW. Increased sensitivity to alcohol induced changes in ERK Map kinase phosphorylation and memory disruption in adolescent as compared to adult C57BL/6J mice. Behav Brain Res. 2012; 230:158–66. [PubMed: 22348893]

Szumlinski KK, Diab ME, Friedman R, Henze LM, Lominac KD, Bowers MS. Accumbens neurochemical adaptations produced by binge-like alcohol consumption. Psychopharmacology (Berl). 2007; 190:415–31. [PubMed: 17225170]

Thomas GM, Huganir RL. MAPK cascade signalling and synaptic plasticity. Nat Rev Neurosci. 2004; 5:173–83. [PubMed: 14976517]

Thorsell A, Tapocik JD, Liu K, Zook M, Bell L, Flanigan M, Patnaik S, Marugan J, Damadzic R, Dehdashti SJ, Schwandt ML, Southall N, Austin CP, Eskay R, Ciccocioppo R, Zheng W, Heilig M. A novel brain penetrant NPS receptor antagonist, NCGC00185684, blocks alcohol-induced ERK-phosphorylation in the central amygdala and decreases operant alcohol self-administration in rats. J Neurosci. 2013; 33:10132–42. [PubMed: 23761908]

Valjent E, Caboche J, Vanhoutte P. Mitogen-activated protein kinase/extracellular signal-regulated kinase induced gene regulation in brain: a molecular substrate for learning and memory? Mol Neurobiol. 2001; 23:83–99. [PubMed: 11817219]

Valjent E, Corvol JC, Pages C, Besson MJ, Maldonado R, Caboche J. Involvement of the extracellular signal-regulated kinase cascade for cocaine-rewarding properties. J Neurosci. 2000; 20:8701–9. [PubMed: 11102476]

Valjent E, Pages C, Herve D, Girault JA, Caboche J. Addictive and non-addictive drugs induce distinct and specific patterns of ERK activation in mouse brain. Eur J Neurosci. 2004; 19:1826–36. [PubMed: 15078556]

Vanhoutte P, Barnier JV, Guibert B, Pages C, Besson MJ, Hipskind RA, Caboche J. Glutamate induces phosphorylation of Elk-1 and CREB, along with c-fos activation, via an extracellular signal-regulated kinase-dependent pathway in brain slices. Mol Cell Biol. 1999; 19:136–46. [PubMed: 9858538]

Wise RA, Koob GF. The development and maintenance of drug addiction. Neuropsychopharmacology. 2014; 39:254–62. [PubMed: 24121188]

Zamora-Martinez ER, Edwards S. Neuronal extracellular signal-regulated kinase (ERK) activity as marker and mediator of alcohol and opioid dependence. Front Integr Neurosci. 2014; 8:24. [PubMed: 24653683]

Zhai H, Li Y, Wang X, Lu L. Drug-induced alterations in the extracellular signal-regulated kinase (ERK) signalling pathway: implications for reinforcement and reinstatement. Cell Mol Neurobiol. 2008; 28:157–72. [PubMed: 18041576]

Zhu Y, Wang Y, Zhao B, Wei S, Xu M, Liu E, Lai J. Differential phosphorylation of GluN1-MAPKs in rat brain reward circuits following long-term alcohol exposure. PLoS One. 2013; 8:e54930. [PubMed: 23372792]

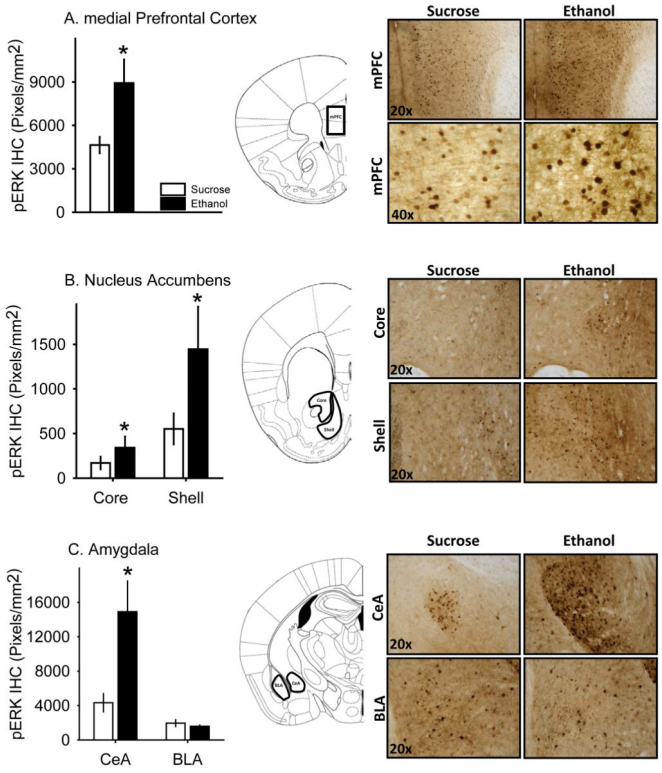

Figure 1. Effect of operant ethanol self-administration on pERK1/2 immunoreactivity

(A–C) *White bars* represent the mean number of immunoreactive pixels/mm^2 for pERK1/2 in tissue taken from mice that self-administered sucrose. *Black bars* represent the mean number of immunoreactive pixels/mm^2 for pERK1/2 in tissue taken from mice that self-administered ethanol. *Vertical lines* represent the SEM for all conditions and *asterisks* denote significance as compared to sucrose ($P<0.05$). Atlas plates adapted from Franklin and Paxinos show the anatomical areas that were analyzed (outlined in black) and representative images of the pERK1/2 immunohistochemistry are shown on the right.

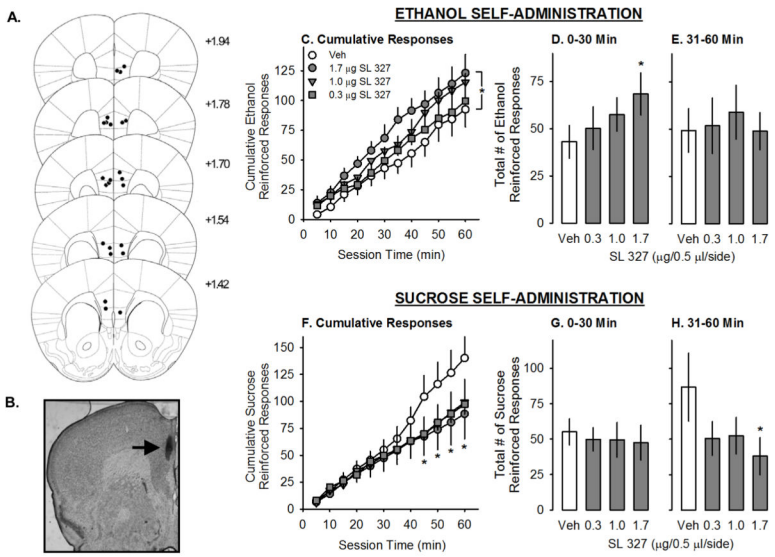

Figure 2. ERK1/2 inhibition in the prefrontal cortex

(A) A schematic representation of the mouse PFC showing A-P distance from Bregma. *Filled circles* indicate the approximate ventral tip of the injection site for each mouse. (B) A representative photomicrograph (2X) of a coronal brain section with a visible injection site (black arrow). (C&F) *White circles* represent the cumulative number ethanol or sucrose reinforced active lever presses after vehicle infusion. *Grey symbols* represent the effect of SL 327 on operant responding for ethanol or sucrose. (D–E&G–H) The *white vertical bars* represent the average number of responses after a vehicle microinjection and the *grey vertical bars* represent the average number of responses after SL 327 microinjection. In all panels, data are presented as mean (*symbols or vertical bars*) ± SEM (*vertical lines*) and *asterisks* denote significance as compared to vehicle ($P<0.05$).

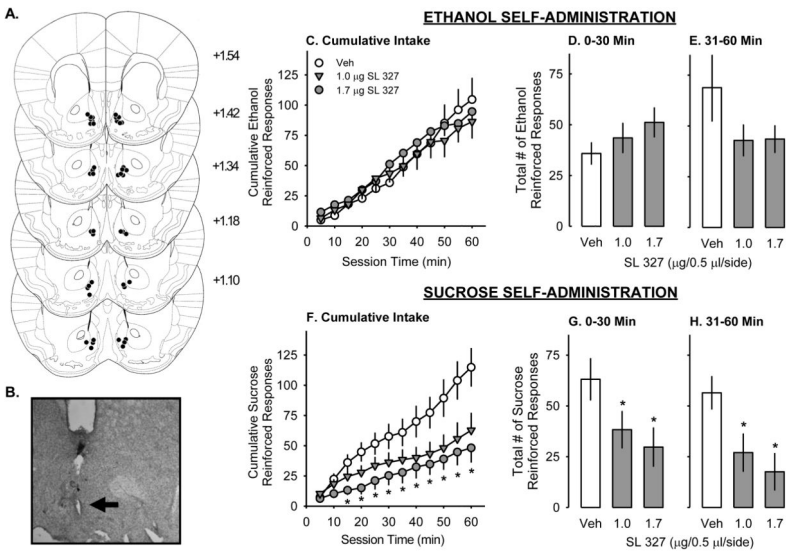

Figure 3. ERK1/2 inhibition in the nucleus accumbens

(A) A schematic representation of the mouse NAC showing A-P distance from Bregma. *Filled circles* indicate the approximate ventral tip of the injection site for each mouse. (B) A representative photomicrograph (2X) of a coronal brain section with a visible injection site (black arrow). (C&F) *White circles* represent the cumulative number ethanol or sucrose reinforced active lever presses after vehicle infusion. *Grey symbols* represent the effect of SL 327 on operant responding for ethanol or sucrose. (D–E&G–H) The *white vertical bars* represent the average number of responses after a vehicle microinjection and the *grey vertical bars* represent the average number of responses after SL 327 microinjection. In all panels, data are presented as mean (*symbols or vertical bars*) ± SEM (*vertical lines*) and *asterisks* denote significance as compared to vehicle ($P<0.05$).

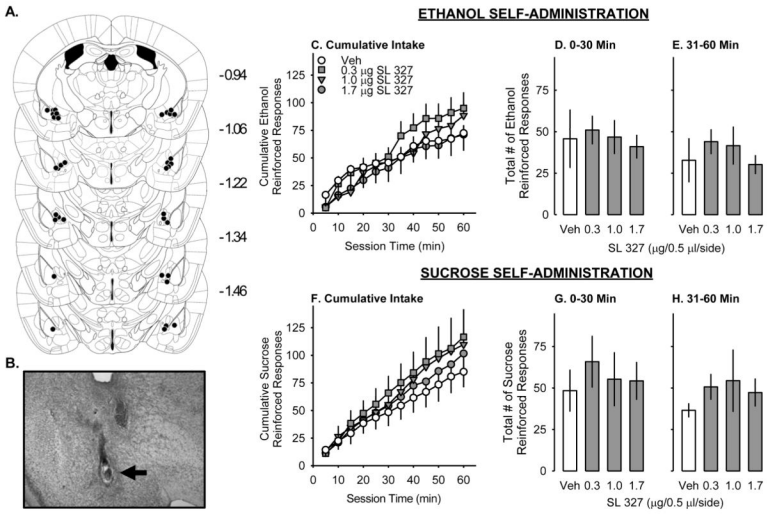

Figure 4. ERK1/2 inhibition in the amygdala

(A) A schematic representation of the mouse AMY showing A-P distance from Bregma. *Filled circles* indicate the approximate ventral tip of the injection site for each mouse. (B) A representative photomicrograph (2X) of a coronal brain section with a visible injection site (black arrow). (C&F) *White circles* represent the cumulative number ethanol or sucrose reinforced active lever presses after vehicle infusion. *Grey symbols* represent the effect of SL 327 on operant responding for ethanol or sucrose. (D–E&G–H) The *white vertical bars* represent the average number of responses after a vehicle microinjection and the *grey vertical bars* represent the average number of responses after SL 327 microinjection. In all panels, data are presented as mean (*symbols or vertical bars*) ± SEM (*vertical lines*).

Table 1

Effect of SL 327 on open field locomotor activity (1 hr session)

PREFRONTAL CORTEX	**ETHANOL DRINKERS**	**SUCROSE DRINKERS**
Vehicle	5922 ± 948	6106 ± 1382
1.0 μg SL 327	6751 ± 932	6307 ± 970
1.7 μg SL 327	5915 ± 727	6252 ± 1615
AMYGDALA	**ETHANOL DRINKERS**	**SUCROSE DRINKERS**
Vehicle	5922 ± 948	6106 ± 1382
1.0 μg SL 327	6751 ± 932	6307 ± 970
1.7μg SL 327	5915 ± 727	6252 ± 1615
NUCLEUS ACCUMBENS	**ETHANOL DRINKERS**	**SUCROSE DRINKERS**
Vehicle	6986 ± 2324	6709 ± 512
1.0 μg SL 327	6722 ± 2084	6817 ± 683
1.7μg SL 327	7674 ± 3198	6911 ± 1155

Table 2

Distinct patterns of drug effect across time and regions

	EtOH Self-Administration		Sucrose Self-Administration	
	0–30 min	*31–60 min*	*0–30 min*	*31–60 min*
PFC	←	↕	↕	→
NAC	Trend for ↑	Trend for ↓	→	→
AMY	↕	↕	↕	↕